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RESEARCH ARTICLE

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PAI-1 transfected-conditioned media promotes osteogenic differentiation of hBMSCs

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Abstract

Reconstruction of injured bone remains challenging in the clinic owing to the lack of suitable bone grafts. The utilization of PAI-1 transfected-conditioned media (P-CM) has demonstrated its ability to facilitate the differentiation process of mesenchymal stem cells (MSCs), potentially serving as a crucial mediator in tissue regeneration. This research endeavored to explore the therapeutic potential of P-CM concerning the differentiation of human bone marrow mesenchymal stem cells (hBMSCs). To assess new bone formation, a rat calvaria critical defect model was employed, while in vitro experiments involved the use of the alizarin Red-S mineral induction test. In the rat calvaria critical defect model, P-CM treatment resulted in significan new bone formation. In vitro, P-CM treated hBMSCs displayed robust osteogenesis compared to the control group, as demonstrated by the mineral induction test using alizarin Red-S. P-CM with hydroxyapatite/β-tricalcium phosphate/fibrin gel treatment significantly exhibited new bone formation, and the expression of osteogenic associated markers was enhanced in the P-CM-treated group. In conclusion, results demonstrate that P-CM treatment significantly enhanced the osteogenic differantiation efficiency and new bone formation, thus could be used as an ideal therapeutic biomolecule for constructing bone-specific implants, especially for orthopedic and dental applications.

KEYWORDS

mesenchymal stem cells, osteogenesis, PAI-1 transfected-conditioned media, tissue regeneration

1 | INTRODUCTION

Tumors, trauma, and infection often cause jaw defects resulting in maxillary and mandibular functional disorders such as facial deformities, tooth loss and occlusal disorders. At present, autologous bone transplantation is the most common treatment method for bone repair and reconstruction. The use of vascularized fibula, ilium and scapula bone flaps requires harvesting autologous bone tissue from other parts of the body, which causes secondary injury and additional pain (Chau & Mobbs, 2009; Danesh-Sani et al., 2017). To resolve the insufficient bone tissue in bone transplantation. There has been a growing interest in tissue engineering, which consists mainly of seed cells, growth factors, and scaffolds (Koch et al., 2009). It is bone marrow mesenchymal stem cells (BMSCs) that are used most commonly in bone tissue engineering, distinguished by their remarkable capacity for osteogenic differentiation. Moreover, BMSCs exhibit a remarkable potential for pluripotent differentiation when exposed to diverse physicochemical environments and cytokine stimuli. These cells can diversify into a plethora of lineages, including osteoblasts, chondrocytes, and even neuron-like cells (Choi et al., 2006; da Silva Meirelles et al., 2006). Consequently, BMSCs hold

vast and promising prospects for applications in tissue engineering, cellular transplantation, andgene therapy.

Plasminogen activator inhibitor-1 (PAI-1) represents a serine protease inhibitor entrenched within the fibrinolytic system inhibitor group (Kaji, 2016). PAI-1 can exert a variety of biological functions, such as acting through fibrinolysis dependent or independent ways to regulate extracellular matrix degradation, cell migration, apoptosis, and cell vascularization (Declerck & Gils, 2013). MSCs differentiate into osteoblasts with the expression of PAI-1 during osteogenic differentiation (Takafuji et al., 2019). Our investigations have elucidated that PAI-1 plays a pivotal role in directing human periodontal ligament stem cells (hPDLSCs) and human periapical follicular stem cells (hPAFSCs) towards differentiation into cementoblasts or odontoblast-like cells (Jin & Choung, 2016; Jin et al., 2015). Recently, conditioned media (CM) from cultured cells rich in secretory factors have been widely used in tissue engineering (Daneshmandi et al., 2020; Ivanisova et al., 2023; Rajan et al., 2017). Earlier studies have noted that P-CM stimulates the osteogenic/cementogenic differentiation of hPDLSCs and hPAFSCs (Jin et al., 2020). The research by Lin further confirms the tremendous potential of growth factors, cytokines, chemokines, and other secretomes present in MSC-CM for clinical applications in periodontal tissue regeneration, providing significant insights for the use of CM in bone tissue engineering (Lin et al., 2021). However, the precise impact of P-CM on osteoblast differentiation and bone formation in BMSCs remains unknown.

In mammals, the transcription factor family known as Nuclear Factor I (NFI) originates from four closely conserved genes, namely NFIA, NFIB, NFIC, and NFIX (Az et al., 1997; Gronostajski, 2000). All four NFI genes find expression in human osteoblasts, with NFIC messenger RNA (mRNA) exhibiting particularly heightened expression in normal osteoblasts compared to other family members (Pérez-Casellas et al., 2009). Osteogenesis is governed by a plethora of transcription factors, including transforming growth factor β (TGFβ), bone morphogenetic proteins (BMPs), Runx2, and Osterix (OSX) (Kobayashi & Kronenberg, 2005; Marie, 2008). Nonetheless, the underlying mechanisms by which these transcription factors orchestrate osteoblast differentiation remain unknown.

In this study, our aim was twofold: to explore the impact of P-CM on the osteogenic differentiation of human BMSCs (hBMSCs) and to unravel the underlying mechanisms involved in this process. Remarkably, we discovered that P-CM significantly fosters the osteogenic differentiation of hBMSCs both in vitro and in vivo. As a result of P-CM treatment, hBMSCs differentiated into osteoblast-like cells, giving rise to bone-like tissues exhibiting high levels of NFIC and OSX expression. These compelling findings strongly suggest that NFIC may play a pivotal role in P-CM-induced osteoblast differentiation.

2 | EXPERIMENTAL SECTION

2.1 | Extraction and culture of hBMSCs

The research protocol, bearing the approval of the Beijing Stomatological Hospital of Capital Medical University, has received ethical endorsement from the esteemed ethics committee (Beijing Stomatological Hospital Ethics Review No. 2011-02), and after obtaining the informed consent of the patients or their guardians, using bone tissue removed during wisdom tooth extraction in adults aged 18–40 for the extraction of hBMSCs. The bone tissue was immersed in α-MEM medium containing antibiotics for 10 min under sterile conditions. The mixture was digested with type I collagenase (3 mg/mL) and neutral protease (4 mg/mL) at 37°C for 2 h, and the tissue suspension was centrifuged at 1100 r/min for 6 min. Resuspend the cell suspension with fresh primary medium (α-MEM medium, 10% fetal bovine serum, 1% penicillin-streptomycin, 1% glutamine) by filtering the cell suspension through a 40-μm filter And inoculated, placed in a 37°C, 5% CO_2 incubator for 1 week. It was observed that the primary cells crawled out from the vicinity of the tissue, and the medium was changed every 2–3 days. The cells were passaged when the cell confluence reached 80%, and the cells of the second to third passages were used for experimental research. To investigate the stemness of isolated hBMSCs, the cells were performed with mesenchymal stem cell specific markers STRO-1 and CD146 primary antibodies (R&D Systems) as described previously (Jin et al., 2015).

2.2 | Immunofluorescence study

To identify the stemness of isolated hBMSCs, the cells were also immuno-stained with STRO-1 and CD146 primary antibodies. The cells fixed with 4% paraformaldehyde and incubated overnight at 4°C with primary antibodies and fluorescent-labeled secondary anti-mouse antibody (Invitrogen). The actin filaments were stained with phalloidin (Invitrogen) and DAPI was used to identify cell nuclei (1:1000 dilution). The cells were visualized using confocal laser scanning microscopy (Olympus).

2.3 | Preparation of PAI-1 transfected-conditioned media (P-CM)

CHO cells were at 1×10^6 cells on 100-mm collagen-coated dishes. When confluence reached 90%, the cells were cultured in Dulbecco's modified Eagle medium differentiation media as described previously (Jin et al., 2020). After 3 days of differentiation, the cells were washed twice with phosphate buffered saline (PBS), differentiation media without fetal bovine serum, and incubated for additional 8 h before the supernatant was collected. After filtration the CM was centrifuged at 1000 rpm for 30 min using a 0.2-μm pore filter (Nalgene), 200 mL of harvested CM were concentrated using ammonium sulfate precipitation and dialyzed against PBS at 4°C. The collected CM was stored at −80°C for subsequent experiments.

2.4 | Cytotoxicity evaluation

The culture-expanded hBMSCs were inoculated into 96-well plates at 1×10^3 cells/well, each well containing 100 μL of medium, and

placed in a 37°C, 5% incubator for 24 h. Then a control group was set up, and different amounts of P-CM were added to the experimental groups, so that the different experimental groups contained 50 or 100 ng/mL of P-CM respectively, with 6 wells in each group. Cells from each group were cultured for 12, 24, 48, 72, 96 h and added to each well in 10 μL of CCK8 reagent, followed by incubation of each group of cells in 96 well plates in an incubator of 37°C, 5% CO_2 for 2 h, the optical density (OD) value at 450 nm was detected using a microplate reader, and the statistical data were presented.

2.5 | In vitro mineral induction study

The hBMSCs were inoculated into 24-well plates at 1×10^5 cells/well, and were divided into 4 groups, with 12 culture wells in each group. The hBMSCs were cultured in the osteoblast induction medium containing 10 mM β-glycerophosphate, 50 μg/mL Ascorbic acid and 10 mM β-glycerophosphate (Sigma Aldrich) for 2 weeks in the presence of 0, 10, 50, and 100 ng/mL P-CM. Detection of the amount of mineralized nodule accumulation was performed on day 14 using 2% alizarin red staining solution, and the OD values were determined for the examined samples.

2.6 | Quantitative real-time PCR (qRT-PCR) analysis

To detect the expression levels of osteogenesis-related genes in hBMSCs of each group, the cells of the control group and the experimental group cultured for 7 and 14 days were extracted RNA by Trizol column method, prepared into complementary DNA, and detected by qPCR. Then, with GAPDH as the internal reference gene, the relative expression of mRNA was evaluated by $2^{-\Delta\Delta Ct}$ method. Table 1 shows the primers that were used for qRT-PCR study.

2.7 | Western blot analysis

The protein levels of Runx2, OSX, and NFIC in hBMSCs cultured in the control group and the experimental group with a concentration of 100 ng/mL P-CM were detected by Western blot on the 1, 4, 7, and 14 days, respectively. After removal of the supernatant, the protein concentration of the cell lysates was determined using a DC protein assay kit. Equal amounts of protein (30 μg/lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with BLOTTO solution and then incubated with anti-pSmad-1/5/8, anti- Runx2, anti-OSX, anti-NFIC and anti-GAPDH overnight at 4°C. It was then probed with horseradish peroxidase conjugated to a secondary antibody for 2 h. Blots were processed using an enhanced chemiluminescence kit and exposed to film.

2.8 | In vivo studies

All research procedures involving animal experiments were carried out in accordance with the Ethics Committee of the School of Stomatology, Capital Medical University (approval number: KQYY-201912-003). For critical-size calvarial defects, 2-month-old male Sprague-Dawley rats ($n = 6$) were used. Absorbable collagen sponges were loaded with human recombinant BMP-2 (2 μg/scaffold; PeproTech) used as a positive control, or vehicle (PBS). Absorbable collagen sponges were also loaded with three concentrations of P-CM (10, 50, and 100 μg/scaffold). For in vivo analysis, P-CM were concentrated by further centrifugation and ultrafiltration using a ultrafiltration centrifuge tube (Millipore) at 8000*g* for 1 h at 4°C. The surgical procedures for the creation of a 5-mm circular defect in the right parietal bone and implantation with the collagen sponges. The rats were euthanized at 4 weeks after surgery, and whole calvaria were fixed in 4% paraformaldehyde for 24 h at 4°C, then analyzed by microcomputed tomographic (μCT) scanning. Each sample was scanned with the Scanner software of Skyscan1174 Micro CT. The rat skull was scanned with a voltage of 50 kV and a current of 800 μA with a scanning resolution of 30.2 μm and a field size of 1304 × 1024. A cylindrical area with a diameter of 5 mm and a thickness of 81 consecutive slices, that is, a cylindrical area with a thickness of about 2.4 mm, was set as the 3D reconstruction region of interest (ROI). Analysis was performed, and bone mineral density (BMD) measurements in the region of interest were performed. The specimens were decalcified with 12% ethylenediaminetetraacetic acid (EDTA) (pH 7.4) for 4 weeks and embedded in paraffin. The samples were sectioned as a thickness of 5 μm and stained with hematoxylin and eosin (H&E), and observed under a light microscope.

TABLE 1 Specified primer sequences used for qRT-PCR analysis.

Gene	Primer sequences
ALP	F: 5′-CCAACTCTTTTGTGCCAGAGA-3′ R: 5′-GGCTACATTGGTGTTGAGCTTTT-3′
OCN	F: 5′-TGAGAGCCCTCACACTCCTC-3′ R: 5′-ACCTTTGCTGGACTCTGCAC-3′
Col 1	F: 5′-CTGACCTTCCTGCGCCTGATGTCC-3′ R: 5′-GTCTGGGGCACCAACGTCCAAGGG-3′
BSP	F: 5′-CAACAGCACAGAGGVAGAAA-3′ R: 5′-CGTACTCCCCCTCGTATTCA-3′
Runx2	F: 5′-CACCATGTCAGCAAAACTTCTT-3′ R: 5′-ACCTTTGCTGGACTCTGCAC-3′
OSX	F: 5′-GCCCACTGGTGCCCAAGACC-3′ R: 5′-CCCGTGGGTGCGCTGATGTT-3′
NFIC	F: 5′-GCTGGACACGACCGACTTC-3 R: 5′-CCGGGACACTTGGATGAGC-3
GAPDH	F: 5′-ACCACAGTCCATGCCATCA-3′ R: 5′-TCCACCACCCTGTTGCTGT-3′

Abbreviation: qRT-PCR, quantitative real-time PCR.

In this transplantation model, P-CM was used to induce osteoblast differentiation of hBMSCs with HA/TCP. The hBMSCs (1.0×10^7cells) were mixed with 100 mg hydroxyapatite/β-tricalcium phosphate (HA/β-TCP) ceramic powder (Zimmer, Inc.), with or without P-CM (50 μg) or PBS, on a 0.5% fibrin gel. The cells were then transplanted subcutaneously into immunocompromised mice ($n = 6$) (NIH-bg-nu/nu-xid; Harlan Sprague Dawley). For histological analysis, samples were obtained at 8 weeks after transplantation and then fixed in 4% paraformaldehyde for 24 h at 4°C. The samples were decalcified with 12% EDTA (pH 7.4) for 4 weeks and embedded in paraffin. Semiserial 5-μm sections were prepared for H&E and immunohistochemical staining and examined under a light microscope.

For immunohistochemistry, deparaffinized sections were immersed with 0.6% hydrogen peroxide for 20 min to extinguish endogenous peroxidase activity. Sections were preincubated with 1% bovine serum albumin in PBS for 30 min and incubated overnight at 4°C with rabbit polyclonal antibodies against BSP, OSX, and NFIC. Sections were incubated with secondary antibodies for 1 h at room temperature and reacted with the avidin biotin peroxidase complex in PBS for 30 min. After staining with 0.05% 3,3′-diaminobenzidine tetrahydrochloride, sections were counterstained with hematoxylin.

2.9 | Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science 13.0 software (SPSS, Inc.). Normal data with equal variance were analyzed using one-way analysis of variance with a Tukey's procedure. Significance was defined as $p \leq .05$. All quantitative data are presented as mean ± *SD*.

3 | RESULTS

3.1 | CM promotes new bone formation in vivo

Using a critical-size calvarial defect model in rats, we assessed the osteogenic capacity of P-CM for bone defect regeneration in vivo. Employing micro CT imaging of the calvarial defects 4 weeks postsurgery. At the defect margins, we observed limited new bone formation in the control group. In contrast, the P-CM-treated group exhibited a substantial and dose-dependent promotion of bone regeneration (Figure 1a,b). To further validate these findings, we performed H&E staining and quantitative histomorphometry, which corroborated the presence of significantly higher amounts of newly regenerated bone in the P-CM-treated group (Figure 1a and 1c). Furthermore, we made a direct comparison between the amount of newly regenerated bone in the group treated with 100 μg of PAI-1 and the BMP-2-treated group. The results demonstrated promising outcomes for the P-CM-treated group, affirming its potential as a potent inducer of bone regeneration.

3.2 | Characterization of hBMSCs

The cultured cells were subjected to flow cytometry with MSCs specific positive markers STRO-1 and CD146. Flow cytometry analysis showed that 90.87% of hBMSCs expressed STRO-1, and 99.6% expressed CD146. Immunoglobulin G was used as an isotype control to exclude the possibility of false positives of the above results (Figure 2a). The morphology of the cells was observed under the microscope, it can be seen that the cells can be adherent at the first day, there are longer protrusions at both ends, and the nucleus is ovoid. Growth speed was slower at the initial stage, increased rapidly after 3rd day, and proliferated to 90% of the dish area by the 7th day (Figure 2b, **a1–a4**). In the immediately following cell immunofluorescence staining, DAPI staining demonstrated a clear blue nucleus, phalloidin staining was tightly and selectively combined with F-actin to show the distribution of the microfilament skeleton in the cells. In hBMSCs, STRO-1 and CD146 expression were stained in green fluorescence and distributed mainly on the surface (Figure 2b, **b1–c4**).

3.3 | The effects of P-CM on the osteogenic differentiation of hBMSCs

To investigate the effect of P-CM on cell viability in vitro, hBMSCs were subjected to treatments with 50 and 100 ng/mL of P-CM. The results obtained from the CCK-8 assay revealed that P-CM exhibited no cytotoxicity at various time points (12, 24, 48, 72, and 96 h), and interestingly, P-CM-treated cells displayed augmented proliferation compared to the control group at 72 and 96 h. Furthermore, no notable distinctions were observed between the different concentrations of P-CM (Figure 3a). Subsequently, we delved into the effects of P-CM on the osteogenic differentiation of hBMSCs in an in vitro setting. Following cultivation of hBMSCs in osteogenic differentiation medium, treated with 0, 10, 50, and 100 ng/mL of P-CM for a duration of 2 weeks, we made significant observations. Notably, P-CM at concentrations of 50 and 100 ng/mL robustly induced osteogenic differentiation, with evident formation of mineral nodules (Figure 3b). Furthermore, the quantification of calcium content post a destaining procedure using Alizarin Red-S staining corroborated these findings, revealing higher calcium levels in the P-CM-treated groups (Figure 3c). After 7 and 14 days, the mRNA expression of osteogenic differentiation markers, such as ALP, OCN, Col1, BSP, OSX, and Runx2, increased significantly, cultured with 100 ng/mL of P-CM compared to untreated cells (Figure 4a,b). Importantly, the expression of *NFIC* was also significantly increased following treatment with P-CM after 7 days, which played critical roles in osteoblast differentiation (Figure 4c). Understanding the fact that

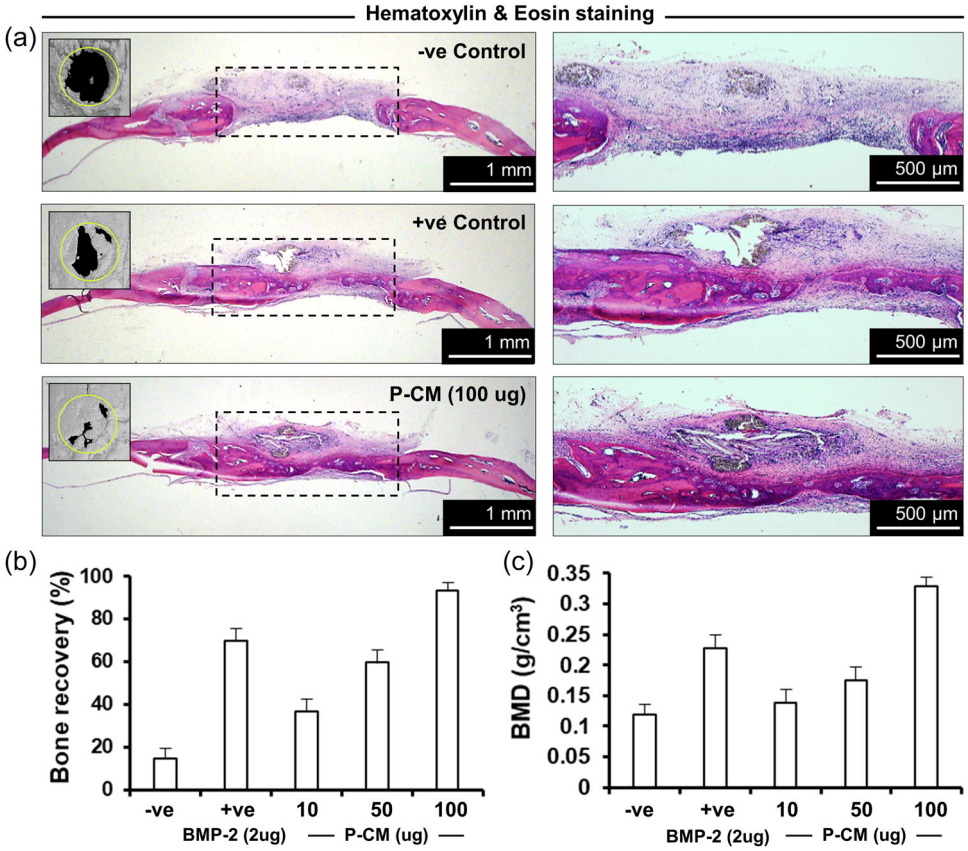

FIGURE 1 Histological analysis of the effects of P-CM on bone regeneration in vivo. (a) Five groups were set up: −ve control group, +ve control group (BMP-2) and P-CM groups with different concentration. After 4 weeks of feeding, CT imaging was performed, followed by sectioning and HE staining. (b) CT calculation showing the bone recovery in each group. (c) Regional bone mineral densitometry using µCT analysis software. µCT, microcomputed tomographic; HE, hematoxylin and eosin.

osteoblastic differentiation using small molecule usually occurred through Smad signaling, we aimed to investigate the early expression of Smad-1/5/8 proteins during osteogenesis. As shown in Figure 4d, the P-CM (100 ng/mL) treated cells were found highly positive for phosphorylated Smad protein (pSmad-1/5/8) than the control group. The p-Smad-1/5/8 expressions was found higher up to 4 days of P-CM treatment and lower expression at 14 days. Surprisingly, NFIC expression was found higher at 7 and 14 days. This suggest that phosphorylation of Smad-1/5/8 may alternatively activate the NFIC, which promote the early accumulation of Runx2 and OSX transcription factors. P-CM significantly increased NFIC expression at 7 and 14 days, and upregulated the levels of p-Smad1/5/8 and Runx2, which act as downstream effectors of BMP-2. Furthermore, P-CM significantly increased OSX expression at 14 days, which act as downstream target of Runx2 in osteogenesis.

3.4 | P-CM/HA/β-TCP promotes new bone formation in vivo

To evaluate the bone-forming potential of P-CM in vivo, we utilized a subcutaneously transplanted model employing immunocompromised mice. The hBMSCs were amalgamated with HA/TCP, with or without the incorporation of P-CM, after that, they were transplanted into immunocompromised mice for 8 weeks. Remarkably, the P-CM-treated group exhibited substantial and noteworthy formation of new bone-like tissue on the surface of HA/TCP, while the control group displayed limited bone-like tissue formation (Figure 5a,b). To further validate the effects of P-CM on osteogenic differentiation, we carried out immunohistochemical staining to assess the expression of osteogenic-associated proteins. Within the newly formed bone-like tissue, the P-CM-treated group demonstrated markedly stronger expression of BSP, the most abundant protein in the bone matrix, in comparison to the control group (Figure 5c,d). Moreover, we observed robust expression of Osterix (OSX), a pivotal transcription factor in osteogenesis, within the newly formed bone-like tissue of the P-CM-treated group (Figure 5e,f). Notably, the expression of NFIC, another critical regulator of osteoblast differentiation, was also prominently elevated in the newly formed bone-like tissue and differentiated osteoblast-like cells in the P-CM-treated group (Figure 5g,h). These compelling findings underscore the potent osteogenic effects of P-CM in conjunction with HA/TCP, emphasizing its promising potential as a facilitator of new bone formation in vivo.

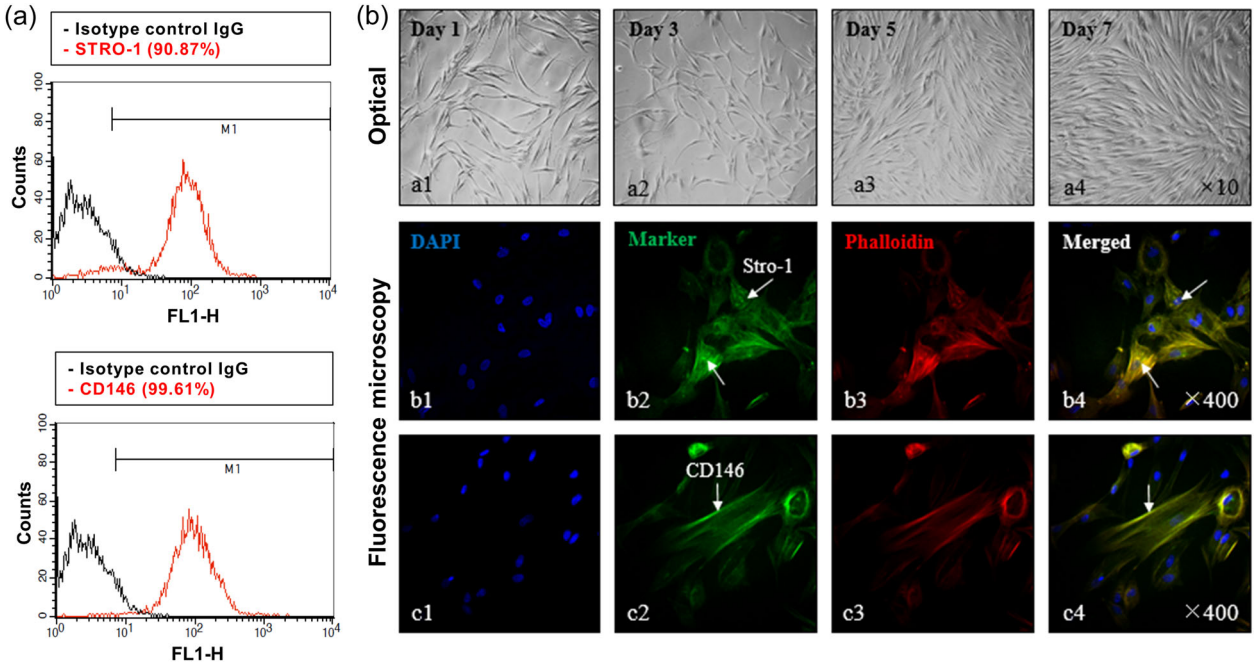

FIGURE 2 Characterization of hBMSCs. (a) The cells were exposed to flow cytometry fluorescence sorting technology using the mesenchymal stem cell markers STRO-1 and CD146. IgG was used as the group control. The M1 gate was set, and the percentage on the right was computed. (b) Microscopic analysis of hBMSCs at indicated time points (a1–a4). STRO-1 (green; b1–b4) and CD146 (green; c1–c4)-based cell immunofluorescence labeling observation (magnification ×40). hBMSC, human bone marrow mesenchymal stem cell; IgG, immunoglobulin G.

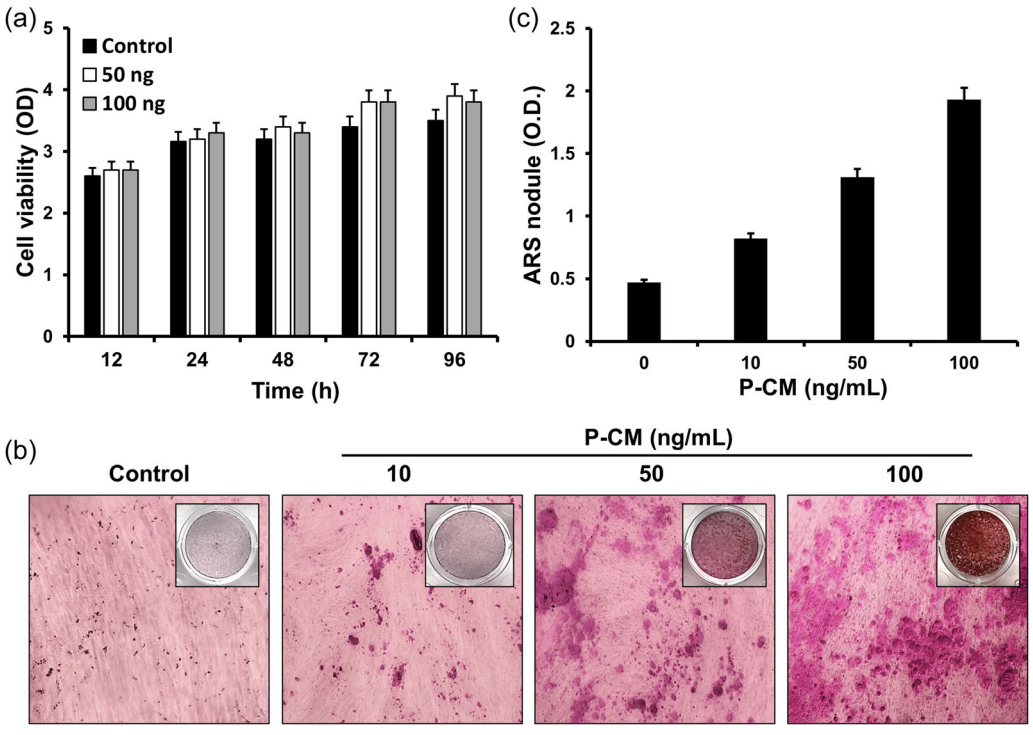

FIGURE 3 Effects of P-CM on osteogenic differentiation of hBMSCs. (a) The cytotoxicity of P-CM on hBMSCs was detected by Cell Counting Kit-8 (CCK-8) assay. The cells were cultured without or with P-CM (50 and 100 ng/mL) at the indicated times. (b) Alizarin red staining was performed on the experimental groups and control group with different concentrations of P-CM after 14 days of mineral induction. (c) The samples were destained to determin the OD value. hBMSC, human bone marrow mesenchymal stem cell; OD, optical density.

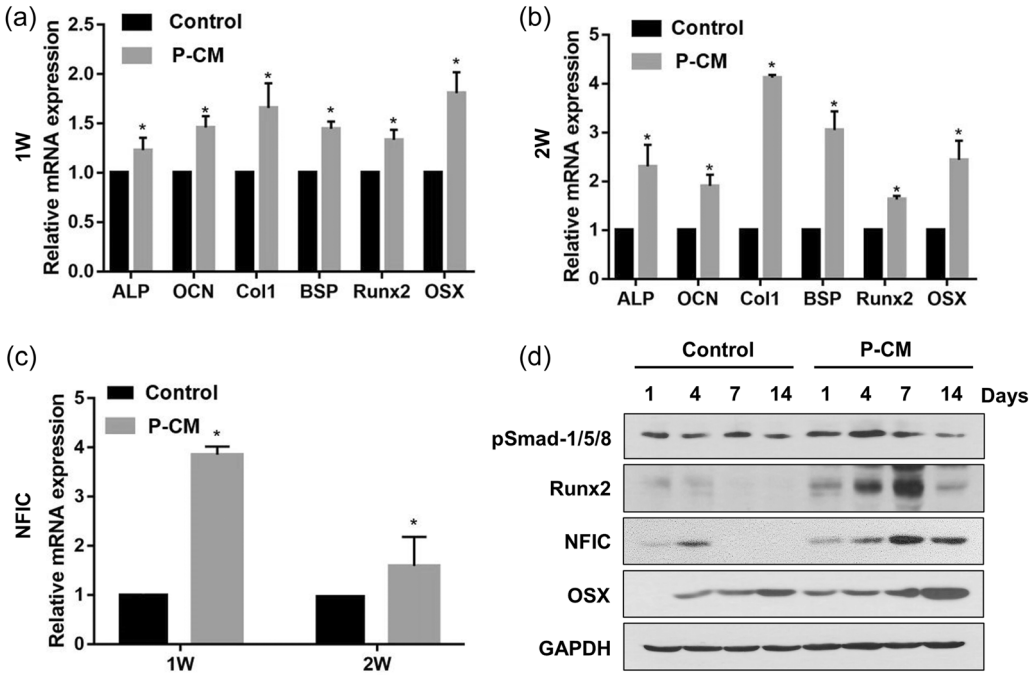

FIGURE 4 The effects of P-CM on the osteogenic differentiation markers. (a–c) RT-PCR was performed on the 7th and 14th day of osteogenic induction to detect the expression of osteogenic-associated genes in the experimental group and control group. $^{*}p < .05$. (d) The protein expression of osteogenic-associated markers was detected by western blot with GAPDH as the internal reference on the 1th, 4th, 7th, and 14th day of the experiment. ALP, alkaline phosphatase; BSP, bone sialoprotein; Col1, type 1 collagen; NFIC, nuclear factor I C; OCN, osteocalcin; OSX, Osterix; RT-PCR, real-time PCR; Runx2, Runt-related transcription factor 2.

4 | DISCUSSION

PAI-1, recognized as a major inhibitor of plasminogen activators, and previous studies have demonstrated its significant regulatory role in bone remodeling, extracellular matrix degradation, cell migration, and apoptosis (Daci et al., 1999; Declerck & Gils, 2013). Our research has further revealed that PAI-1 promotes the differentiation of PDLSCs into cementoblasts and PAFSCs into odontoblasts (Jin & Choung, 2016; Jin et al., 2015).

CM is frequently utilized in diverse areas of regenerative medicine and holds therapeutic potential for bone repair in humans (Kumar et al., 2019; Vizoso et al., 2017). The utilization of CM presents several advantages and challenges, which are outlined below. Advantages of CM usage for bone repair include its regenerative potential, as it contains various bioactive molecules such as growth factors, cytokines, and extracellular vesicles. These components can significantly promote tissue regeneration and bone repair. Additionally, the risk of immune response is minimized when using autologous CM derived from the patient's own cells, reducing the likelihood of rejection or adverse reactions. Furthermore, simplified application is possible with ready-made solutions that can be easily stored and administered in a clinical setting. Hurdles and Challenges in Using CM for Bone Repair: Limited Understanding of Composition: The exact composition of CM can vary based on the source cells and culture conditions, making it challenging to standardize its formulation and dosage. Variable Efficacy: The efficacy of CM for bone repair can vary between different studies and preparations, making it difficult to establish consistent results.

Recently, we reported that P-CM induces osteogenic/cementogenic differentiation in Dental derived mesenchymal stem cells (Jin et al., 2020). However, the specific effects of P-CM on osteogenic differentiation in hBMSCs have remained unexplored until now. Our current study unveils compelling evidence that P-CM not only promotes bone formation but also induces osteoblast differentiation in hBMSCs, an effect mediated by the upregulation of NFIC and OSX. These findings imply that P-CM could act as a stimulus for MSCs to differentiate into osteoblasts cell types under diverse conditions.

Our in vivo findings were in line with the in vitro results, as P-CM consistently facilitated the differentiation of hBMSCs, leading to increased mineral nodules formation. Furthermore, we observed that P-CM positively influenced the mRNA expression of key osteoblast markers, such as ALP, OCN, Col1, BSP, Runx2, OSX, and NFIC, which are all associated with osteoblast differentiation. ALP serves as a hallmark protein product indicative of osteoblast phenotype and differentiation, and it is widely recognized as a crucial osteoblast marker. OCN, appearing at the final stage of osteoblast differentiation, plays a vital role in regulating calcium ion homeostasis and bone mineralization through its ability to bind to $Ca2^{+}$. Col1, a specific collagen synthesized by osteoblasts, accounts for 90% of the organic matrix in bone tissue, and its high content reflects the maturation status of osteoblasts. BSP, a member of the sibling gene family, is significantly associated with osteoblast differentiation and the initiation of

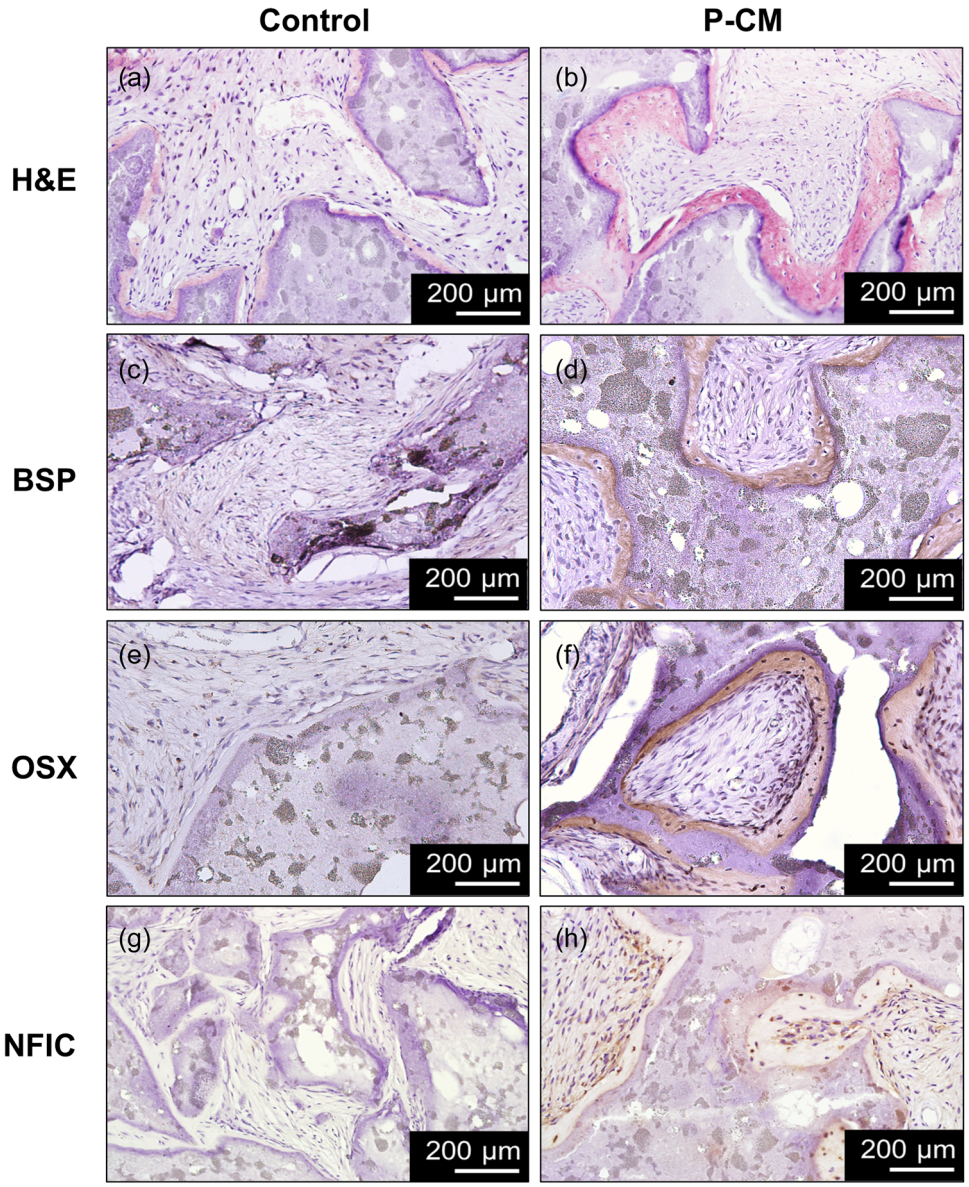

FIGURE 5 Histological analysis of the effects of P-CM on bone formation. (a, b) H&E staining of generation of bone-like tissue in the control group and the P-CM-treated group (50 μg). (c–h) The immunohistochemistry of the new generated bone area of the P-CM-treated group and the control group after 8 weeks, using BSP, OSX, and NFIC respectively. Under a 200 μm microscope. H&E, hematoxylin and eosin.

mineralization (An et al., 2016; He et al., 2011; Liu & Lee, 2013; Long, 2011). Furthermore, the master transcription factor, Runx2, is essential for the regulation of osteogenic differentiation in MSCs (Komori, 2006).

BMP-2 is recognized as a powerful growth factor that promotes osteoblast differentiation and induces bone formation, it is known to be a powerful growth factor (Chen et al., 2012; Wan & Cao, 2005), We hypothesize that P-CM acts as a downstream effector of BMP-2 to affect cells. Our results showed that P-CM treatment led to increased expression of p-Smad-1/5/8, Runx2, and OSX, indicating the activation of the BMP-2 signaling pathway. Runx2-/-mice (Komori et al., 1997), known to lack bone formation, further underscore the significance of Runx2, which is essential for inducing major bone matrix genes in immature osteoblasts (Komori, 2010). Moreover, OSX expression, regulated by BMP-2 signaling through both Runx2-dependent and -independent mechanisms, is considered a pivotal transcription factor in osteogenesis (Nishio et al., 2006). Based on these findings, Runx2 and OSX might be involved in osteogenic differentiation of hBMSCs induced by P-CM. Notably, we observed that P-CM treatment also increased NFIC expression. The NFI gene family plays vital roles in both prenatal and postnatal development, and each member of the family serves various essential functions. Among them, NFIC acts as a critical regulator of postnatal growth and plays a significant role in the proliferation of adult progenitor cells (Plasari et al., 2010). NFIC$^{-/-}$ mice, while displaying normal prenatal bone development, exhibit defects in postnatal bone growth, indicating its crucial role in osteoblast differentiation (Lee et al., 2014). It means that NFIC is also an essential

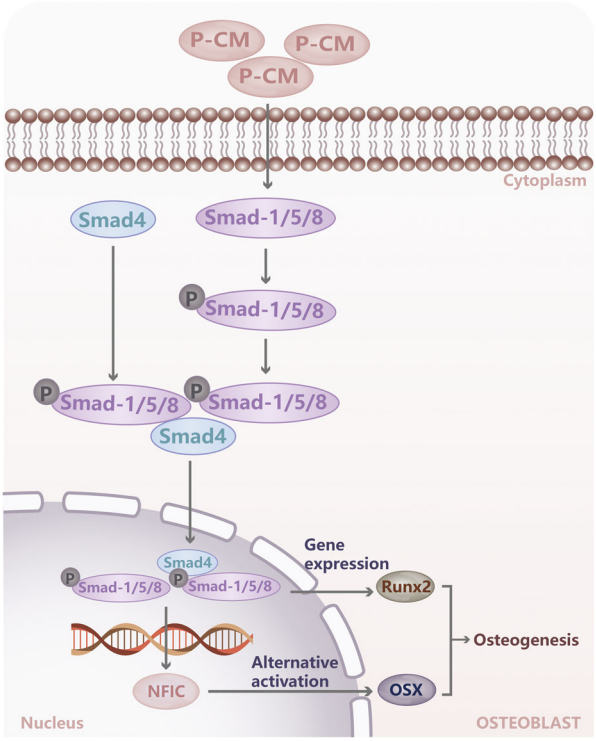

FIGURE 6 A hypothetical diagram showing the possible alternative pathway of osteogenesis in hBMSCs via NFIC activation through Smad-1/5/8 signaling in the presence of P-CM. hBMSC, human bone marrow mesenchymal stem cell.

transcription factor, which regulates osteoblast differentiation. However, further investigations are necessary to unravel the functional relationships among the transcription factors Runx2, OSX, and NFIC during osteogenic differentiation. We have proposed a hypothetical diagram (Figure 6) that presents the potential alternative activation of NFIC through p-Smad-1/5/8 in hBMSCs under P-CM treatment.

The balance between biochemical and mechanical microenvironments is crucial for effective tissue regeneration, scaffolds capable of providing proper. Scaffolds that provide appropriate mechanical support are essential for osteogenic differentiation (Komori et al., 1997; Nakashima et al., 2002). In our previous studies, we have shown that the combination of PAI-1 with dentin matrix and HA/TCP leads to enhanced biological activities. This combination effectively induces the differentiation of periodontal ligament stem cells (PDLSCs) into cementoblasts, facilitating the regeneration of cementum on the surface of the dentin matrix (Jin et al., 2015). HA/TCP has proven to be effective in inducing hard tissue formation in MSCs, resulting in the generation of several hard tissues, including bone, dentin, and cementum (Arinzeh et al., 2005; Ohgushi et al., 1990). In our study, we combined P-CM with HA/TCP and hBMSCs and transplanted them subcutaneously into immunocompromised mice. The results revealed P-CM's ability to induce hBMSCs to differentiate into osteoblasts and form bone tissues. Notably, we observed significant expression of the bone-specific marker, BSP, in the newly formed bone tissues of the P-CM-treated group. Additionally, we observed robust expression of OSX and NFIC in the differentiated osteoblasts and bone tissues of the P-CM-treated group. These findings suggest that P-CM may induce osteogenic differentiation through the NFIC-OSX pathway. However, further investigations are necessary to fully understand the functional relationship between NFIC and OSX during osteoblast differentiation and bone formation.

5 | CONCLUSION

In conclusion, our findings demonstrate that P-CM effectively promotes bone formation in vivo, with NFIC playing a critical role as a new transcription regulator in P-CM induced osteogenic differentiation of hBMSCs. The upregulation of OSX and NFIC during osteoblast differentiation further supports the potential therapeutic applications of P-CM in bone and tooth development. Overall, our study provides novel insights into the transcriptional regulation and osteogenic potential of P-CM, paving the way for future research in bone and tooth tissue regeneration.

AUTHOR CONTRIBUTIONS

Ki-Taek Lim and Hexiu Jin: conceived and designed the experiments. **Zhang Li, Hou Kegui, and Wang Piao**: performed the experiments. **Hou Kegui and Wang Piao**: mainly responsible for animal experiments. Hou Kegui, and Wang Xuejiu: wrote the draft manuscript. **Ki-Taek Lim and Hexiu Jin**: revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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